Immune-Epithelial Interactions Exacerbate Cell Injury During Airway Reopening Through Changes in Cell Spreading

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Abstract

During the acute respiratory distress syndrome (ARDS), bacterial/viral infections, including COVID-19, significant pulmonary edema and ARDS patients often require mechanical ventilation (MV) for survival. Unfortunately, MV is known to exacerbate lung injury due to high mechanical forces through reopening of flooded alveoli. In this study we subjected lung epithelial cells to airway reopening forces in-vitro under conditions including co-culturing with an immune cell found in the lung called macrophages. We found that macrophages cause a significant increase in cell death during airway reopening, likely due to a contact dependent mechanism. We then show that macrophages cause a decrease in epithelial cell density. We then used computational modeling to confirm that lower cell packing results in higher tangential strain on the epithelial cell membrane. We also saw that the over-expression of miR-155 decreases this macrophage exacerbated cellular injury.

Introduction

Mechanical ventilation (MV) is an important life support strategy to maintain adequate blood oxygenation in patients with acute respiratory distress syndrome (ARDS). Unfortunately, high mechanical forces generated during MV are known to induce lung injury (1). For example, during ARDS, alveolar-capillary barrier dysfunction leads to fluid accumulation within small airways and alveoli. The reopening of these fluid-occluded lung regions involves the repeated propagation of an air-liquid interface over epithelial cells lining airway/alveolar walls. Due to an elevated surface tension, this cyclic airway/alveolar reopening exerts large mechanical stresses to epithelial cells and causes significant cellular injury known as atelectrauma (2). Atelectrauma

results in cell necrosis, barrier disruption and inflammation (3). Although our group has used novel in-vitro systems to model atelectrauma in-vitro and tested potential cytoprotective agents (4), these studies did not account for multicellular interactions present within pulmonary alveoli. One important cell native to distal lung airways and alveoli is the alveolar macrophage (AM). This cell resides directly on taqop of epithelial cells and communication between epithelial cells and macrophages is known to be important in various diseases (5). However, the role of AMs in regulating or contributing to ventilation induced lung injury (VILI) is vastly understudied and to our knowledge, no one has investigated how AM may influence atelectrauma in alveolar epithelial cells.

Several studies have investigated how changes in cell morphology and structure alter cell injury during airway reopening. We have previously shown that softer and more fluidized cells undergo less injury during cyclic airway reopening due to the cell's ability to dampen rapidly applied hydrodynamic forces [6,7]. During atelectrauma the hydrodynamic forces applied by the microbubble are not constant. The forces are rapidly applied and removed, causing the forces to be time dependent. Therefore, cells that are more fluid-like may not have time to respond and deform to these rapid forces. This lack of deformation prevents membrane deformation, inhibiting cell necrosis. Additionally, computational and in-vitro studies have shown that cells with a decrease in height/width and height/length ratio experience less injury during reopening. It has been shown that flattened cells with reduced variation in topography experience significant reduction hydrodynamic stress from the air-liquid interface [8]. Investigation into how macrophages alter cell injury through changes in cell mechanics may elucidate potential treatments to reduce VILI.

A promising therapeutic for VILI has been extensively studied in our lab: MicroRNAs

(MiRNAs). MiRNAs are short non-coding regions of RNA that bind to and prevent transcription of mRNA. MiRNA can be modulated by increasing miRNA expression with miRNA mimics. In the last few years, the use of microRNAs for treatment of diseases has shown great promise and is rapidly evolving, regulating 60% of genes [9]. MiRNAs are a key regulator in many biological pathways and are often dysregulated in disease, including inflammation as seen in lung injury. MicroRNAs target multiple cellular networks which can allow it to alter entire cellular pathways in disease. For example, MircroRNA-155 has shown to regulate the rho kinase pathway which plays an important role in cellular structure [10]. Therefore, we will deliver mir-155 mimic to A549 epithelial cells to alter cell morphology and mechanical properties to reduce airway reopening induced injury. MicroRNAs are advantageous over other reagents due to their innate aspect and are already normally found in biological systems. Delivery of microRNA may be key to reducing lung injury and improving outcomes in ARDS patients.

Methods

1. **Cell Culture**

Human A549 epithelial cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal bovine serum and 1% antibiotics/antimycotics mixture. A549 Cells were grown in standard culture conditions at 37°C and 5% CO_2. Cells were seeded onto 40 mm coverslips in 60 mm dishes for 4 days to achieve a confluent monolayer. THP-1 monocytic cell lines were maintained in RMPI, with 10% FBS, and 1% antibiotics/antimycotics. THP-1 cells were treated with 10-100nM PMA for 48 hours to induce differentiation. Following differentiation, THP-1s were seeded onto A549 cells at a 1:5 ratio and co-incubated for 24 hours. THPs expressing green fluorescent protein were also used to measure cell specific death and THP-1 removal during flow. Alternatively, human alveolar epithelial cells were grown in human

epithelial cell cultures with 10% Fetal bovine serum and 1% antibiotics/antimycotics mixture for 4 days. Subsequently, human primary alveolar macrophages were seeded onto primary human alveolar cells for 24 hours.

2. **THP-1 conditioned Media Collection.**

As described above, THP-1 cells were differentiated with 10nM PMA and subsequently cultured in serum-free RPMI media for 48 hours. After 48 hours the media was collected and centrifuged for 5 minutes at 1200 RPM to remove cells from the supernatant. A549 cells were then treated with the conditioned media or standard serum-free RPMI media for 24 hours prior to the experiment.

3. **Airway Reopening Simulation**

As described previously (3) a Bioptechs FCS2 chamber (Bioptechs, Butler, PA) and a programmable PHD 2000 syringe pump were used to simulate cyclic airway reopening. Phosphate buffered solution was used to fill the channel at 30mm/s. In this study we simulated 5 reopening events with a reopening velocity of 3 mm/s in a 500 um high channel. We then used fluorescent microscopy to perform a live/dead stain to measure cell viability. 10 images were taken along the center of the channel.

4. **Cell Morphology and Mechanics Characterization**

Using fluorescence imaging, the effect of macrophages on epithelial actin formation was evaluated. Cell actin and nuclei were stained with phalloidin and DAPI respectively. Fluorescent images were used to characterize epithelial cell size and cell density.

5. **Computational Modeling of Lung Injury**

The COMSOL finite element package was used to develop a sophisticated fluid-structure interaction computational model of cellular deformation during air-liquid flows. Unlike previous models[4] this model simulates the dynamic interaction between air-liquid interfacial flows and cellular deformation. We used this model to investigate how the changes in cell density alters maximum tangential strain in the plasma membrane. The model parameters matched the in-vitro valued. The air-liquid interface velocity was set to 30mm/s in a 500μM height channel.
The capillary number for water/air interface was calculated as 4.3×10^{-4}.

Cell Transfection

After 72 hours incubation in 60 mm dishes, A549 cells were transfected in Opti-MEM medium with miR-155 mimic for 24 hours. After 24 hours the media was replaced with DMEM. The A549 cells were then co-cultured with THP-1 macrophages or monocultured for another 24 hours before the airway reopening experiment.

Results

1. **Macrophages Modulate Epithelial Cell Injury during airway reopening**

   As shown in figure 1A-D, 24 hour co-culture of macrophages with A549 epithelial cells results in an increase in injury during airway reopening simulation. The addition of THP-1 macrophages results in an increase in epithelial cell death from 15% to 32%, compared to monoculture of A549 epithelial cells alone. A similar effect is seen in primary cells taken directly from living tissue. Human alveolar epithelial cells (HAEC) experienced an increase in cell death from 16% to 29% when co-cultured with primary human alveolar macrophages (Fig 1D).

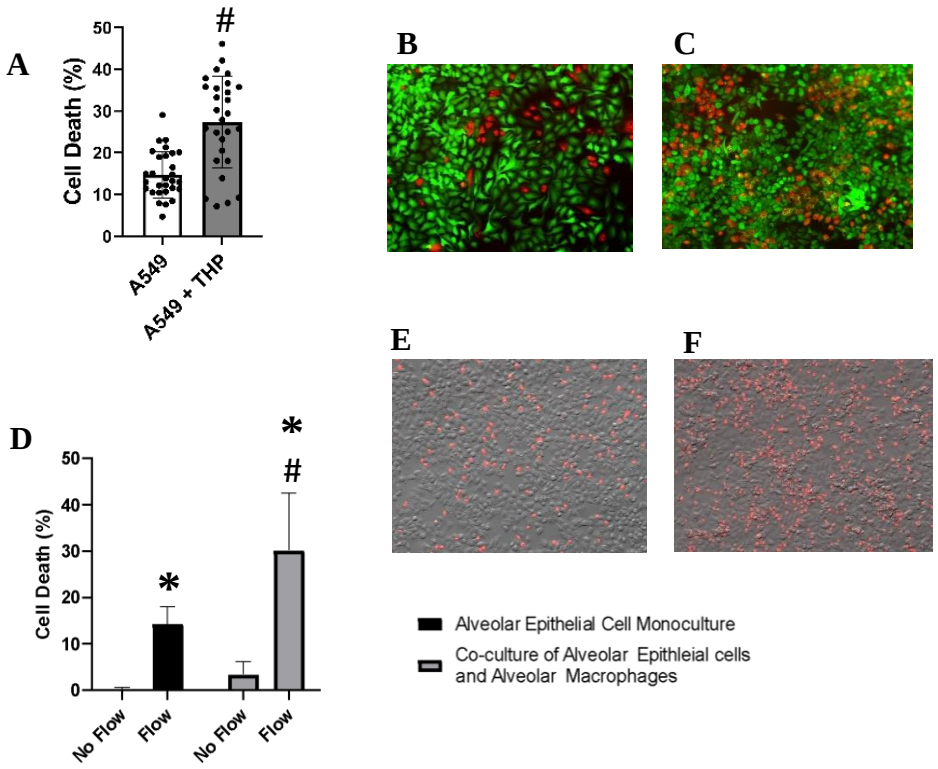


Figure 1: Human alveolar epithelial cells showed higher rate of death in co-culture with macrophages than monoculture ($p<.05$) after simulated airway reopening in (A) immortalized cell lines and (D) primary cell lines. (C,D) representatve image of live/dead assay after atelectrauma in monoculture (B) and co-culture conditions (C).
(E,F) representiative images after atelectrauma of primary cells monoculture (E) and co-culture conditions (F). * indicates significant difference with respect to control no flow conditions ($p<.05$). # indicates a significant difference in cell death to monoculture flow conditions ($p<.05$).

2. Cell injury primarily occurs in A549s during co-culture flow simulation

Approximately 50% of THP-1 macrophages are removed during simulated airway reopening.

99% of cell death that occurs during airway reopening, occurs in A549 epithelial cells, compared to 1% occurring in THP-1 cells (Fig 3B).

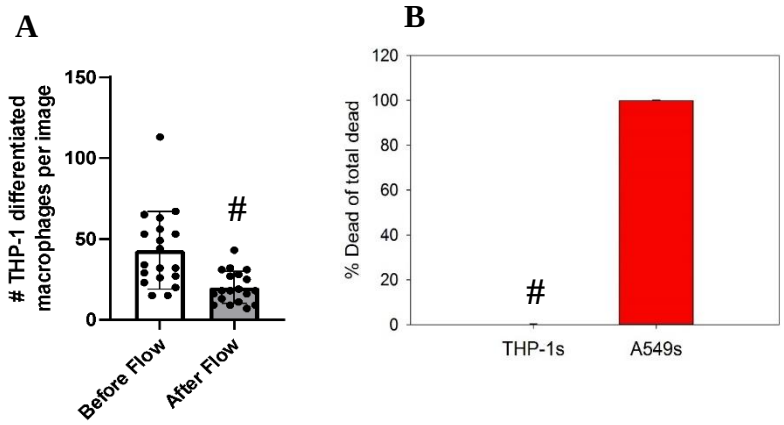


Figure 2: (A) Approximately 50% of macrophages are removed after 5 simulated airway reopening events (B) Majority of death after co-culture and atelectrauma occurred in epithelial cells (99%) ($p<.05$). # indicates significant difference to control

3. Macrophages Modulate Cell Death through Contact Mechanism

Interestingly, A549 cells cultured in the presence of 100 ng/ml TNF-α or macrophage conditioned media did not have a significant increase in cell death during atelectrauma compared to A549 cells cultured in normal growth medium.

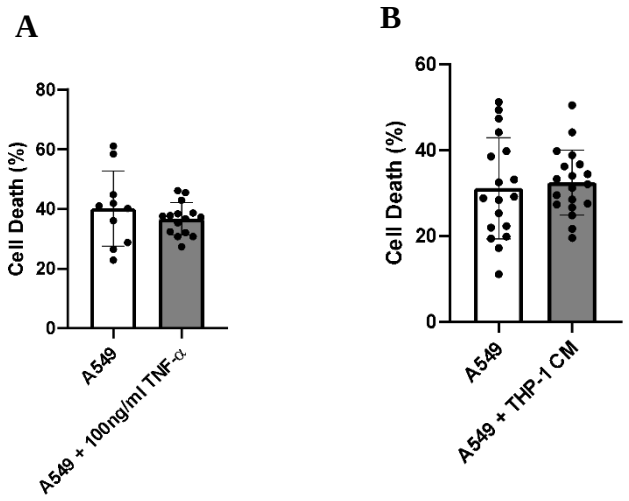
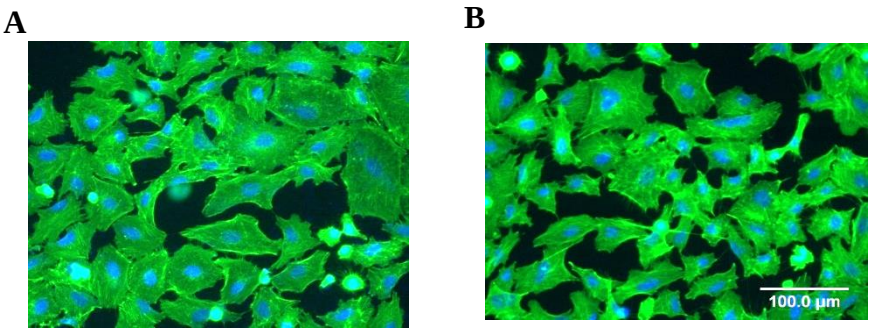


Figure 3: A549s treated with 10nM PMA differentiated THP-1 conditioned media or 100ng/ml TNF-α for 24 hours did not have significantly different cell death than A549s in serum free media (p>.05)

4. Macrophages Effect on Cell Spreading

Live cell staining indicates higher cell packing in monocultures of A549 cells and lower cell density in co-culture with macrophages (i.e. 65 epithelial cells per image vs 56 cells per image in co-culture conditions). There is also a significant increase in the average area each cell covers in the dish. A549 epithelial cells in co-culture have an area of 1800μm^2 compared to 1500μm^2 in monoculture conditions.



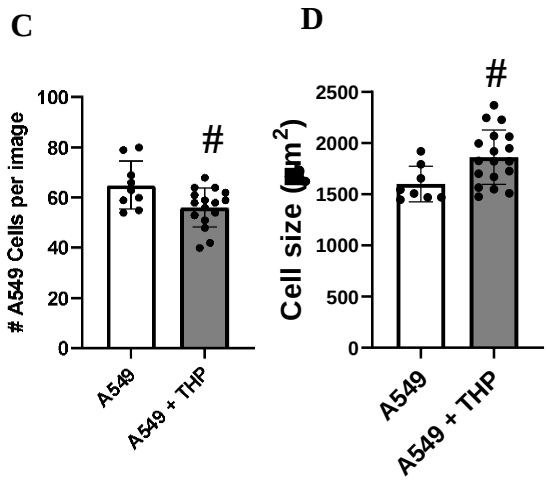


Figure 4: Cell actin fibers (green) and cell DNA (blue) were stained with phalloidin and DAPI respectively in A549 monoculture (A) or A549 and THP-1 macrophage co-culture (B) Co-culture cells showed a decrease in cell density (C) Co-cultured cells showed an increase in cell area. # indicates significant difference ($p<.05$)

5. Cell Density Modulates Tangential Strain

Computational models indicate that air-liquid flows over cells that are tightly packed experience a smaller maximum tangetial strain on the plasma membrane. This indicates that more densely grown cells exhibit less deformation and plasma membrane strain than cells that are more dispersed.

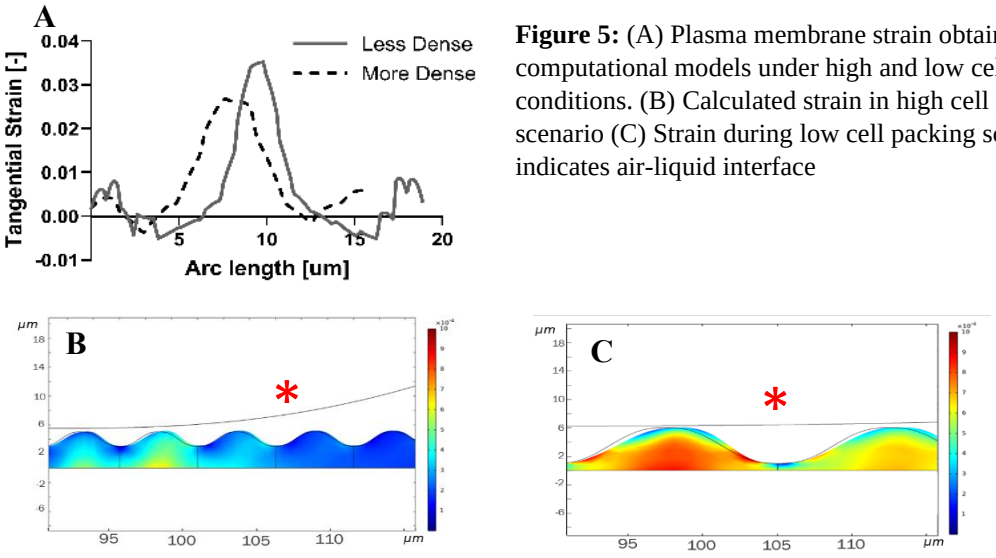


Figure 5: (A) Plasma membrane strain obtained from computational models under high and low cell packing conditions. (B) Calculated strain in high cell packing scenario (C) Strain during low cell packing scenario. * indicates air-liquid interface

6. Delivery of miR-155 Decreases Cell Injury

Delivery of miR-155 to A549 cells 48 hours prior to airway reopening injury resulted in a significant decrease in epithelial cell death. It also significantly reduced cell death in co-culture conditions as well.

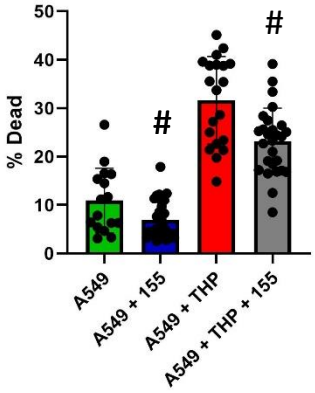


Figure 6: A549 cells were treated with miR-155 mimic or miR-scramble for 24 hours prior to co-culture or monoculture conditions for a further 24 hours before trauma # indicates significant difference compared to scramble miR-treatment ($p<.05$)

Discussion

A549 cells saw an increase in cell death during co-culture conditions; however this effect is not seen during co-culture in THP-1 conditioned media which contains all the factors produced by THP-1 cells. Furthermore the addition of TNA-α, a main inflammatory factor produced by macrophages, did not cause a significant increase in cell death. We also saw that most cell death occurred in A549 cells (>99%). This confirms the addition of macrophages does not increase cell death due to an increase in cells that are vulnerable to mechanical stress injury. Overall, this data suggests that the presence of macrophages increases cell death in alveolar epithelial cells during lung reopening, likely due to cell-cell contact dependent mechanisms. Using fluorescent staining we also examined the effect of macrophages on the morphology and confluence of epithelial cells. During co-culture conditions epithelial cells experience decreased cell density, increased cell size, and increased cell death. This suggests that macrophages alter the confluence and the morphology of epithelial cells to cause a change in the mechanical forces and cell injury in lung epithelial cells. The computational data shows more dense cells have a lower tangential strain.

This suggests cells are more shielded when packed closer together. This is a novel distinct mechanism from literature showing increased cell protuberance increases pressure gradients (11). We then observed that delivery of miR-155 to A549 cells prior to co-culture conditions mediated the macrophaged exacerbated cellular injury. Therefore, miR-155 treatment may decrease lung injury through mechanical property modulation.

To further elucidate the mechanism by which macrophages alter cellular injury, future studies will include probing epithelial mechanical properties such as stiffness and viscoelasticity using atomic force microscopy. Acute respiratory distress syndrome is one of the main causes of mortality in COVID-19 patients, and frequently requires ventilation (12). This study may have implications in how to mitigate ventilator induced lung injury in ventilated patients. miR-155 treatment may be key to altering the mechanical properties of cells in the lung, reducing ventilator induced lung injury.

Acknowledgments
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